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STUDIES ON THE DEVELOPMENT OF THE SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*. III. EMBRYONIC SYNTHESIS OF CILIARY PROTEINS

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The formation of cilia at the blastula stage represents an important morphological event in the development of echinoderm and other embryos. Parthenogenetic activation leads to normal (but haploid) division, blastula formation, subsequent ciliogenesis, and indeed to full larval development and metamorphosis, indicating that maternal genetic information and organelles are quite sufficient for full development, and that the male nucleus, centriole, and basal body are superfluous (*cf.* Wilson, 1925). Parthenogenetic activation of enucleate half-eggs of the sea urchin leads to cytokinesis, blastula formation, and hatching, but to no further development, indicating that maternal cytoplasmic information is sufficient for at least the mechanics of pre-gastrular development (Harvey, 1940). The formation of cilia during enucleate parthenogenetic development has been reported (Harvey, 1940), but this highly important claim has not yet been substantiated by electron microscopy. Early development through the point of cilia formation and hatching takes place in the presence of sufficient actinomycin D to totally abolish RNA synthesis, indicating a stable masked messenger RNA template (Gross, Malkin, and Moyer, 1964; Guidice, Mutolo, and Donatuti, 1968). After "deciliation", normal regeneration of cilia can occur in the presence of Actinomycin D (Auclair and Siegel, 1966), without DNA-dependent RNA synthesis. These same workers also demonstrated that multiple cilia regenerations could take place in the presence of puromycin although the synthesis of ciliary proteins was reduced by 90%, thus indicating an extensive pool of ciliary precursors at least at the gastrula stage. The repeated formation of cilia in the near absence of transcription and translation would point to a self-assembly process from pre-existing active precursors. Pulse-labeling of developing sea urchin embryos and the subsequent isolation of cilia indicate a fairly steady rate of total ciliary protein synthesis prior to and early in ciliogenesis (Auclair and Meismer, 1965; Stephens, unpublished). If cilia formation is a self-assembly process and if the ciliary proteins are made prior to ciliogenesis, what then triggers this precisely-timed morphological event? Is some critical component synthesized immediately prior to organelle assembly in such small amount that it is overshadowed by the bulk proteins of the cilium, or is there some initiating enzymatic activation process whereby modification of the pre-existing components brings about a "crystallization" of all of the requisite proteins into a cilium?

This present report attempts to answer some of these questions through a fractionation of ciliary axonemes, obtained from pulse-labeled sea urchin embryos, into known structural components, followed by an analysis of the sequence of synthesis of such components.

Materials and Methods

Embryonic material

The sea urchin *Strongylocentrotus droebachiensis* was obtained from the Supply Department of the Marine Biological Laboratory. Gametes were shed, egg jelly coats were removed, and the eggs were fertilized and grown at 7.5° ± 0.2° C according to the methods outlined in full elsewhere (Stephens, 1972a). All operations were conducted at or below the growth temperature to avoid temperature shock and assure synchrony. Sterile sea water containing 0.05% sulfadiazine was used to initially wash the eggs and was used in all subsequent egg suspensions.

Experimental design

In a typical experiment, 12 ml of dejellied eggs from one female were fertilized in 250 ml of sea water at zero time. An aliquot of egg suspension containing 1 ml of eggs was immediately withdrawn, spun down in a hand centrifuge, and 5.0 ml of sea water containing 4 μc/ml of ^{14}C-leucine were added. This incubation mixture was shaken gently during a three hour pulse period. At the end of this period, the eggs were spun out of the pulse solution, resuspended in 10 ml of sea water chase containing 1% cold leucine, and agitated for an additional 30 minutes. The embryos were again gently spun down, resuspended in 100 ml of normal sea water, and allowed to develop undisturbed through cilia formation and hatching. Additional aliquots were taken at successive 3 hour intervals through 30 hours of growth and all treated in an identical manner. The net result of this protocol was to produce 11 aliquots of ciliated blastulae, differing only in the time-point of pulse labeling. With adequate temperature control, all embryos hatch within a 15–30 minute interval.

Ciliary axoneme preparation

Cilia were isolated 12 hours after hatching by transferring the blastulae to 10 ml of ice-cold hypertonic sea water (containing an additional 30 g/l NaCl, Auclair and Siegel, 1966) and stirring gently for 2 minutes. The deciliated embryos were immediately centrifuged and resuspended in fresh normal sea water for regeneration studies. The supernatant from the deciliation was centrifuged at 10,000 × *g* for 10 minutes to sediment the cilia. The resultant pellet was suspended in 5 ml of 1% Triton X-100 detergent containing 30 mM Tris HCl, pH 8.0, and 3 mM $MgCl_2$ (Stephens, 1970a) for 30 minutes to remove the ciliary membranes, matrix, and any accidental cell debris. The ciliary axonemes were then sedimented at 10,000 × *g* for 10 minutes and the supernatant was discarded. Phase-contrast microscopy of the pellet (Fig. 1) indicated only the presence of ciliary axonemes; no cell fragments or bacteria were observed. Regenerated

cilia were isolated in an identical manner from the above resuspeneded deciliated embryos after an 8-hour regeneration period.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

To each ciliary axoneme pellet produced above, 0.2 ml of 1% sodium dodecyl sulfate (SDS) containing 10 mM phosphate, pH 7.0, 1% 2-mercaptoethanol, and 10% glycerol was added and then heated to 90° C for 2 minutes. Gel electrophoresis was carried out by the methods of Shapiro, Vinuela, and Maizel (1967) on 100 microliter duplicate aliquots of the dissolved axoneme sample (initial volume of about 0.3 ml) applied to 10 cm length 5% acrylamide gels and run at

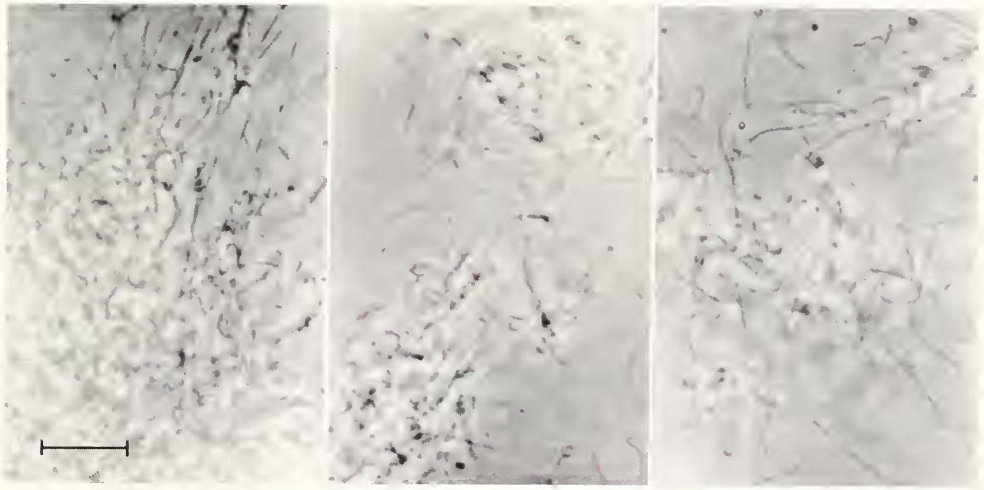

FIGURE 1. Phase-contrast micrographs of Triton X-100 extracted ciliary axonemes under oil immersion Leitz optics. Removal of the ciliary membrane causes aggregation of the axonemes. Scale marker equals 10 microns.

50 volts. The gels were stained for 2 hours with at least 10 volumes of Fast Green (Gorovsky, Carlson, and Rosenbaum, 1970) at a concentration of $\frac{1}{2}$% in 50% methanol-10% acetic acid (Stephens, 1972b), and then diffusion-destained by multiple changes of 7% acetic acid. Parallel gels of known molecular weight proteins (bovine serum albumin, tubulin, actin, and dynein) were run to calibrate the system. Bovine serum albumin applied to parallel gels in 5, 25, 50, and 75 microgram amounts stained in accord with Beer's Law over this range; ciliary axonemes were always applied at a concentration less than 50 micrograms. The analytical gels were then either photographed or else subjected to direct microdensitometry with a Joyce-Loebl MK III double beam recording densitometer. The amount of protein applied to each gel, as judged either from direct protein determination or from integrated microdensitometer tracings of stained gels, was identical within the limits of reproducibility of these methods (±15%).

Protein concentration

Total embryonic, ciliary, or axonemal protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard. Determination from SDS-solutions was carried out by initially dissolving the sample in the *absence* of 2-mercaptoethanol, removing an aliquot of 25 microliters for protein determination, and then adding the mercaptoethanol from a micropipette prior to heating the sample. After electrophoresis, relative proportions of proteins in mixtures were estimated from the microdensitometer trace of a stained gel, assuming equal color yield for all components.

Autoradiography

After microdensitometry, the gels were sliced longitudinally, dried *in vacuo*, and autoradiographed for 2 weeks on Kodak No-Screen X-ray film, exactly according to the procedures of Fairbanks, Levinthal, and Reeder (1965). By using the densitometric tracings of the unsliced gels of labeled and stained ciliary axonemes, the amount of protein and radioactivity applied to each gel, and the densitometric traces of the corresponding autoradiogram, the relative specific activities of the various major components could be easily calculated. Changes in activity were estimated by comparison of traces at different time points.

Scintillation counting

Radioactivity in the SDS-solutions of isolated axonemes and in the ^{14}C-leucine supernatant solutions before and after pulse-labeling were determined by counting 25 microliter aliquots in Beckman "Fluoralloy" liquid scintillation fluid, using a Beckman LS 250 counter. Appropriate corrections were made for efficiency.

Isotope

Uniformly-labeled ^{14}C-L-leucine (Schwarz-BioResearch) with a specific activity of 312 mc/mmole was diluted to a concentration of 4 μc/ml in sterile sea water and neutralized to pH 8.0. The final leucine concentration was 1.3×10^{-2} μmole/ml. For each pulse, 20 μc of total isotope was used per ml of eggs or roughly per 100 mg of total egg protein.

Data reproducibility

The entire experimental design, involving uptake data, pulse-chase labeling of first-generation cilia, regeneration of cilia from previously-labeled embryos, gel electrophoresis, and autoradiographic analysis, was carried out on two separate egg batches, one early and one late in the breeding season. The protocol yielded duplicate gels, each of which in turn produced duplicate gel slices for autoradiography; comparable time points yielded essentially superimposable densitometric tracings and both experiments yielded identical patterns of sequential synthesis.

Results

The overall pattern of embryonic and ciliary protein synthesis

Throughout embryogenesis beyond the first division, between 75% and 88% of the total added ^{14}C-leucine was incorporated into protein of the whole embryo, as determined by counting TCA-precipitated protein from either an aliquot of the post-chase embryos or of the ciliated embryos after hatching. No measurable difference could be demonstrated in these two cases, indicating the effectiveness of the chase (*cf.* Auclair and Siegel, 1966). Measurement of the label left in the

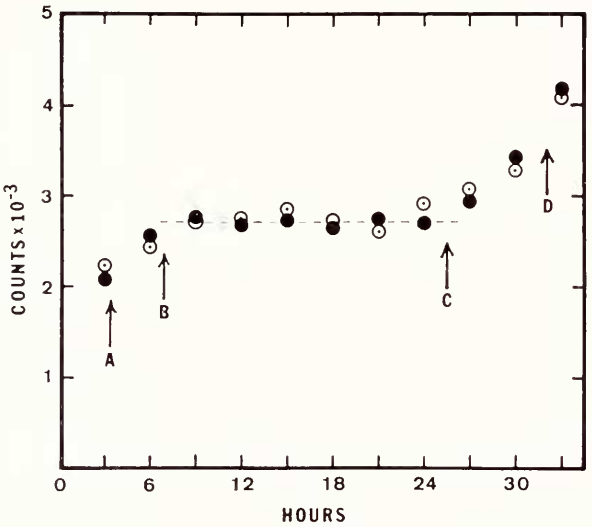

FIGURE 2. Incorporation of ^{14}C-leucine into ciliary axonemes after three-hour pulses. Various developmental stages are noted as follows: A—first division; B—8-cell stage; C—cilia formation begins; D—hatching. Specific activity is in counts per minute per microgram of axonemal protein. Experimental points are from two separate sets of experiments.

pulse-label supernatant fluid yielded a value of 88–92% uptake for the three-hour pulse periods in all but the first division time point. During the first division, 80–83% of the label was taken up and about 75% of the amount of total embryonic protein was synthesized relative to the later divisions.

The isolated ciliary axonemes reflected this overall synthetic pattern, except during the process of ciliogenesis itself. Thus during the major portion of the early developmental process, regardless of the time point at which the pulse was introduced, the axonemes isolated after hatching had essentially the same specific activity. The only variation was an expected increase to this constant level after the first division and also a marked increase prior to hatching (Fig. 2). Thus the bulk of axonemal proteins are made before ciliogenesis as reported previously (Auclair and Meisner, 1965), but there is a marked increase in synthesis during the morphogenetic process. When whole cilia are counted after a similar pulse-labeling experiment, this increase is much less obvious; this is probably due to

a masking effect since the cilia are at least twice as "hot" as the axonemes, contain twice as much protein, and this protein (chiefly membrane, matrix, and cell debris) is of nearly constant activity.

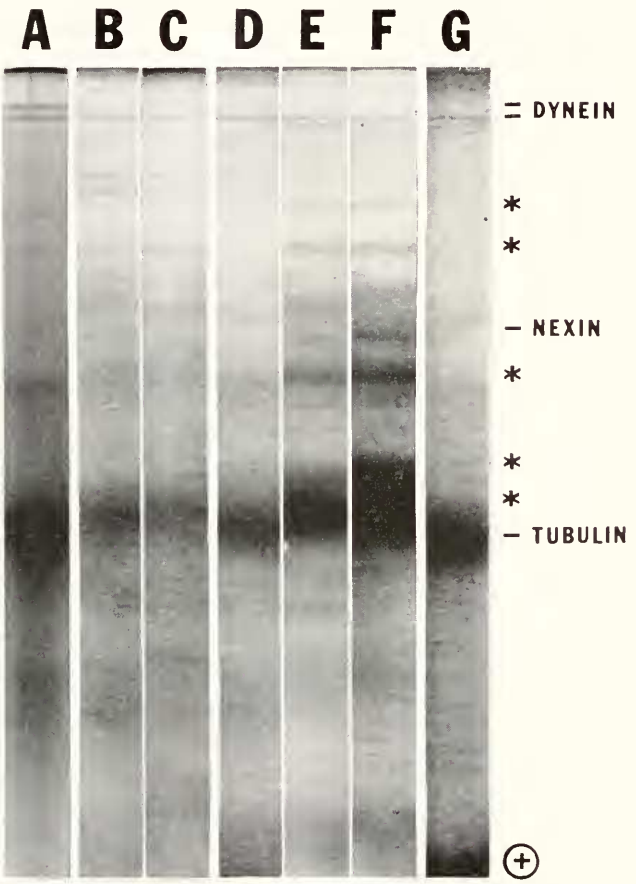

FIGURE 3. Acrylamide gel autoradiograms of ciliary axonemes. A—stained gel, 27–30 hour pulse period; B and C—autoradiograms of 6–9 hour and 12–15 hour pulse period gels; D, E, and F—autoradiograms of 24–27 hour, 27–30 hour, and 30–33 hour pulse period gels, respectively; G—autoradiogram of gel from regenerated axonemes from the 27–30 hour pulse period. Slight misalignment of bands is due to uneven drying of gels. Several bands are virtually indetectable in the stained gel, but are apparent in the autoradiogram due to high specific activity resulting from *de novo* synthesis (*cf.* Fairbanks *et al.*, 1965). All gels were loaded with the same amount of axonemal protein.

Over the moderately constant range of ciliary protein incorporation, the axoneme represents about 1.5% of the total incorporated radioactivity but accounted for only about 0.08% of the total embryonic protein by actual measurement after isolation. A very rough calculation of the expected amount of total axonemal protein, based on one 30 μ cilium per cell per 500-cell blastula in a sample of

125,000 blastula, gives a value of 0.1%. Thus during the pre-ciliogenesis period, the specific activity of the axonemal proteins average about 15–20 times higher than that of the embryonic proteins as a whole. Nearly ten-fold higher whole cilia values were reported by Auclair and Siegel (1966) for cilia regenerated from late gastrula.

Synthesis of specific proteins of the "9 + 2" structure

The data of Figure 2 would not indicate much in the way of a dramatic change in ciliary protein synthesis prior to or early in ciliogenesis, an event occuring at about 26 hours and thus within the 24–27 hour pulse. During active ciliogenesis, (the 27–30 hour pulse period) the specific activity increases by nearly 25% and during hatching, (the 30–33 hour pulse period) the specific activity increases to a value roughly 55% over the pre-ciliogenesis level. As noted above, and by previous workers, the bulk of the ciliary proteins are made prior to the actual formation of the organelle; the present data indicate a marked increase in incorporation during ciliogenesis. The question now becomes one of determining what these various proteins are and when they are synthesized. Analysis of SDS-electrophoretic autoradiograms of identical amounts of ciliary axonemes from embryos differing only in the time-point of the pulse-label shows that the proteins of the "9 + 2" axoneme fall into four general classes with regard to their timetable of synthesis.

Figure 3 includes photographs of autoradiograms for 6–9 hour and 12–15 hour pre-ciliogenesis pulse periods, 24–27 hours (representing initiation of cilia formation), 27–30 hours (active ciliogenesis), and 30–33 hours (representing final stages in ciliogenesis prior to and during hatching). Figure 4 is a composite diagram of autoradiographic microdensitometer tracings for 9–12 hour and 21–24 hour pre-ciliogenesis pulses and the 24–33 hour period of active ciliogenesis. These diagrams should be referred to in regard to the results discussed below.

(1.) *Pre-existing proteins*—The ciliary ATPase *dynein* (Gibbons, 1965 and 1966) is composed of two high molecular weight components in 2:1 ratio (Linck 1970 and 1971). Having revised molecular weights of 500,000 and 460,000 as determined through linear 3% acrylamide-SDS gel electrophoresis (Linck, 1971), these two prominent bands are easily observed on stained gels (Figs. 3A and 4, bottom trace). Upon autoradiographic analysis of such gels *no label is ever detected in the higher molecular weight dynein band*, regardless of pulse-label time. Weisenberg and Taylor (1968) report a 13S dynein-like ATPase protein in unfertilized urchin eggs, while Stephens (1972b) has detected only a *single* higher molecular weight band in the dynein region when whole eggs are subjected to SDS-acrylamide gel electrophoresis. The former authors speculated that the 13S ATPase may be a ciliary precursor; the protein synthetic data presented here indicates that it has to be since it is not synthesized after fertilization. Other minor components may also pre-exist but their amount would be too small to detect under the present methodology.

(2.) *Proteins synthesized at a constant pre-ciliogenesis rate*—As implied above, the *lower molecular weight component of dynein* is synthesized after fertilization; its rate of synthesis does not change even during the most active periods of cilio-

genesis, as evidenced by its near-constant height and area in all of the autoradiograms (Fig. 4, all traces). Fractionation of ciliary axonemes into two equally active dynein fractions, one lacking the lower molecular weight component, would indicate that this component is structural rather than enzymatic (Linck, 1971 and in preparation). The *tubulin* fraction is also synthesized at a uniform rate beyond the first division; during active ciliogenesis, however, the estimated specific activity of total tubulin increases by about 50% (Table I). Fractionation of doublet tubules into A- and B-components (Stephens, 1970a) or electrophoretic separation by charge (Bryan and Wilson, 1971) indicates no specific activity difference between tubules or subunits. A similar conclusion was drawn by Raff, Greenhouse, Gross, and Gross (1971) for subunits of the total tubulin fraction from fertilized sea urchin eggs, separated as a vinblastine precipitate from cell homogenates taken at successive time points during development.

TABLE I

Relative tubulin specific activity in virgin versus *regenerated cilia as an approximate measure of synthesized pool size at any given time point, determined from two separate experiments by approximation of area under the tubulin peak in autoradiographic densitometer traces from samples of known specific activity*

Pulse time	Virgin	Regenerate	% of original in regenerate
30–33 hr	1265/1215	1200/1160	95
27–30	1200/1120	1140/1100	96
24–27	800/775	545/515	67
21–24	860/820	625/590	72
18–21	800/760	540/590	66
6–9	815/785	570/530	68
0–3	575/525	445/405	77

Other major components that remain constant prior to and during ciliogenesis have molecular weights of 280,000, 105,000, 88,000, 50,000, 45,000, 38,000, and 32,000. The latter two components vary somewhat in position and quality since the 5% gel system was designed for maximum resolution in the higher molecular weight region. The 105,000 and 50,000 or the 88,000 and 45,000 molecular weight components may be related as monomer to dimer, and thus only two components rather than four may exist. The 32,000 molecular weight material has been tentatively identified as an *adenylate kinase* on the basis of ammonium sulfate fractionation, enzyme determination, and electrophoretic identification of the most active fraction. Regardless of identity of these minor components, it should be obvious that the bulk of the structural proteins, *i.e.*, these plus tubulin and dynein, do not show any differential synthesis during early development. Since they represent at least three-fourths of the total axonemal protein, the observation of near-constant synthesis of "ciliary" proteins prior to and early in ciliogenesis is easily rationalized. It is the initiation of synthesis of *minor* constituents that marks the beginning of ciliary morphogenesis.

(3.) *Proteins apparently synthesized* de novo *at ciliogenesis*—Since it is difficult to determine pre-existing pools for minor ciliary constituents and nearly im-

possible to detect a very low but constant rate of pre-ciliogenesis synthesis for such components, the term *de novo* must be used here with caution. In the sense of this study, it may only mean that a marked increase takes place. Regardless of terminology, rapid synthesis of such proteins is of obvious morphogenetic significance.

The proteins that are synthesized at a high rate uniquely at ciliogenesis can be best discussed by molecular weight. Six major components with molecular weights of about 250,000, 200,000, 165,000, 130,000, 73,000, and 68,000 can be readily detected, particularly on the 30 hour pulse tracing (Figs. 3–4; marked with stars). The 250,000, 73,000, and 68,000 molecular weight components are detectable on the 24 hour pulse tracing. The 165,000 and 130,000 molecular weight proteins appear in the 27 hour pulse, while the 200,000 molecular weight component is only obvious in the 30 hour pulse tracing. These differences may simply reflect the relative amounts of these constituents in the axoneme rather than any real differential or sequential synthesis; this appears to be the case for the first detectable components at least. Shorter and more frequent pulses coupled with large scale isolation and very accurate quantitation would be needed to prove sequential or differential synthesis; such is beyond the scope and intent of this current qualitative study.

In sea urchin sperm flagellar axonemes, a 165,000 molecular weight protein has been identified as the linkage material connecting the nine outer doublets together and has been named *nexin* (Stephens, 1970b); ciliary axonemes of other species show additional 80,000 and 240,000 molecular weight bands when subjected to similar nexin isolation procedures. It is possible that in this study the components at approximate molecular weights of 73,000, 165,000 and 250,000 may all be the linkage protein nexin, related as monomer to dimer to trimer. Whatever the interpretation, these and the remaining *"de novo"* components, by a process of elimination, must be either spoke or linkage proteins in the basic "9 + 2" structure. It is not illogical to expect that the assembly of an organelle might be initiated by the synthesis of such vital architectural elements.

(4.) *Proteins with decreased synthetic rates*—This category is the most equivocal, since detection is borderline. One major protein band with a molecular weight of about 300,000 is very prominent on the stained gels. It shows a substantial rate of synthesis on all of the early gels but at 24 hours and beyond it is much less detectable. It is unclear whether it actually decreases in synthesis or the background of other proteins has increased relative to it. This component is also likely to be related to radial or circumferential structural elements. Limited synthesis of such a structural component might offer a useful mechanism for limiting either the length or the number of cilia that an embryo forms at any given time.

Regeneration of cilia from previously pulse-labeled and deciliated embryos

Two major ciliary components (a dynein band and tubulin) and many minor ones are made continuously at constant rate, while at least six other structural protein bands are apparently synthesized *de novo* at and during ciliogenesis. The relative specific activities of these components in regenerated cilia from the original deciliated embryos compared to that of the virgin cilia should give some clue as to the relative amount of each of these constitutents that was synthesized during the pulse period. Does the cell initially manufacture only the amount of material

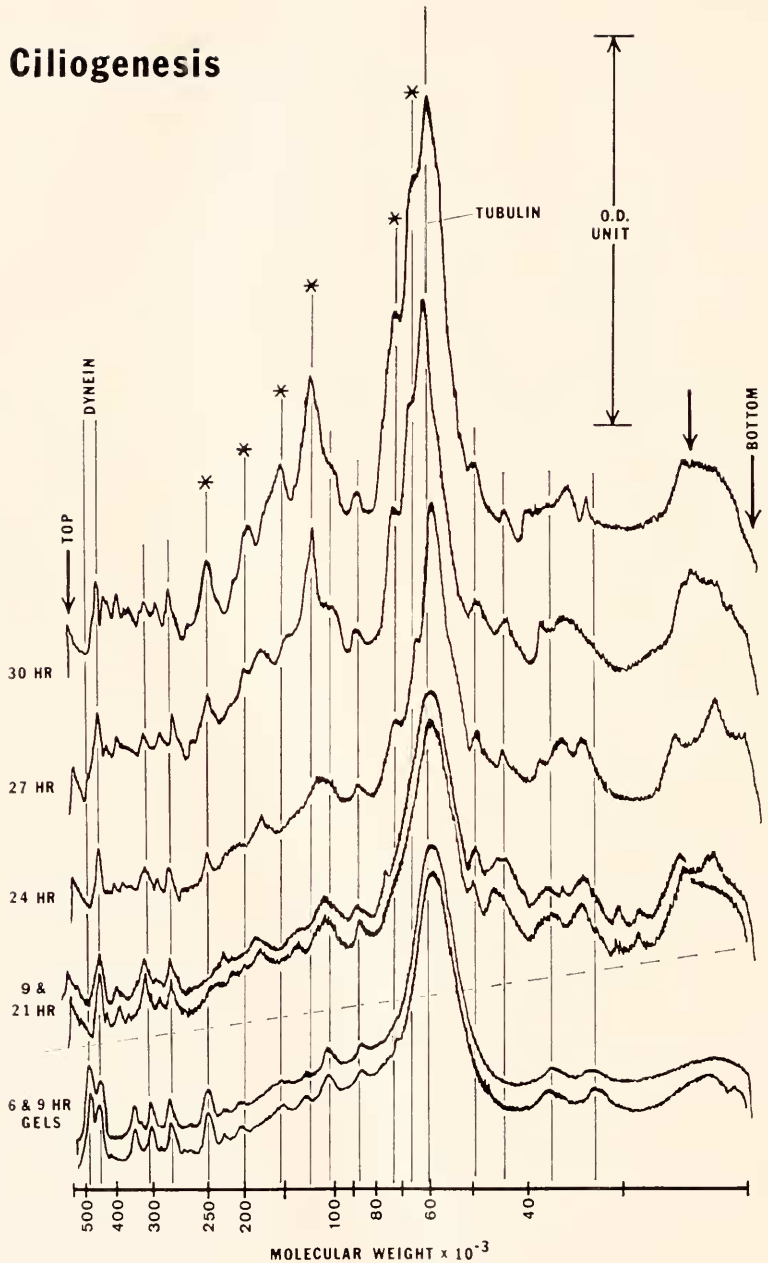

FIGURE 4. Incorporation of ^{14}C-leucine into specific axonemal proteins from embryos labeled at varous times. The microdensitometer tracings were made from gels such as those of Figure 3. The bottom pair of traces are from stained gels of axonemes from 6–9 hour and 9–12 hour pulse periods; the remainder are 9–12 and 21–24 hour pre-ciliogenesis pulse periods, and 24–27 hour, 27–30 hour, and 30–33 hour pulse periods of active ciliogenesis.

that it needs for one generation? Experiments by Auclair and Siegel (1966) indicated that a moderately large precursor pool is available to cilia regenerating at the late gastrula stage, but what is the magnitude of the pool synthesized prior to and during the initial ciliogenesis?

Figure 3G is an autoradiogram of regenerated cilia from an initial 27–30 hour pulse followed by deciliation and should be compared with Figure 3E, the virgin cilia of this same time point. Figure 5 includes representative autoradiographic microdensitometer tracings of regenerated cilia from the same time points illustrated in Figure 4 and includes a superimposed 30–33 hour trace of virgin cilia for comparison.

Microdensitometric analysis of autoradiographic gels from regenerated cilia of previously labeled and deciliated embryos reveal several interesting facts. The dynein band, the general background material in the molecular weight range of 90,000 to 250,000, and the lower molecular weight material below 50,000 have essentially the same specific activity in regenerated cilia as in the original harvest, within an experimental error of ±15%. This could imply that a relatively large and constant pool of these materials has been made prior to deciliation, or simply that whatever has been made will be incorporated into the cilia until the (limited) supply is gone, assuming of course no appreciable synthesis of these components after deciliation. Thus both a large pool with low post-deciliation synthesis or a limited pool utilized totally are consistent with the data.

The tubulin, on the other hand, showed a somewhat decreased specific activity in the regenerates *versus* the virgin cilia in all time points prior to ciliogenesis. In early development, the regenerates have only about three-fourths of the original counts, while the regenerates from embryos pulse-labeled during ciliogenesis have over 95% of the original specific activity (Table I). Assuming that tubulin synthesis continues after deciliation at a rate at least comparable to that during early development, and therefore that most of the tubulin lost to the first harvest is replaced with cold tubulin, the data would indicate a pool three to four times larger than that needed for one generation exists prior to ciliogenesis. This somewhat crude estimate is not unlike that of Auclair and Meismer (1965) who have demonstrated 4 regenerations in the near-absence of protein synthesis. The pool *during* ciliogenesis cannot be estimated accurately by this method since the decrease in specific activity of regenerates *versus* virgin cilia during the active process is within the limits of error; there can be no question that it is even larger, however.

Quite significantly, the *"de novo"* proteins uniquely synthesized during ciliogenesis barely appear in the autoradiograms of the regenerates whose parent embryos were labeled during this period (Fig. 5, top trace). These previously-prominent components are reduced to 10–20% of their original values, a fact indicative of the production of only one "round" of these apparently critical components since essentially all of the protein labeled during ciliogenesis was isolated with the virgin cilia. The striking thing about the autoradiograms of the regenerated cilia is the fact that all pulse points look the same, with the minor exception

Unmarked arrow indicates the position of the tracking dye front. Asterisks mark the components apparently synthesized *de novo*. The dashed line is an averaged tracing from blank gel-autoradiograms, giving a combined film and instrumental baseline.

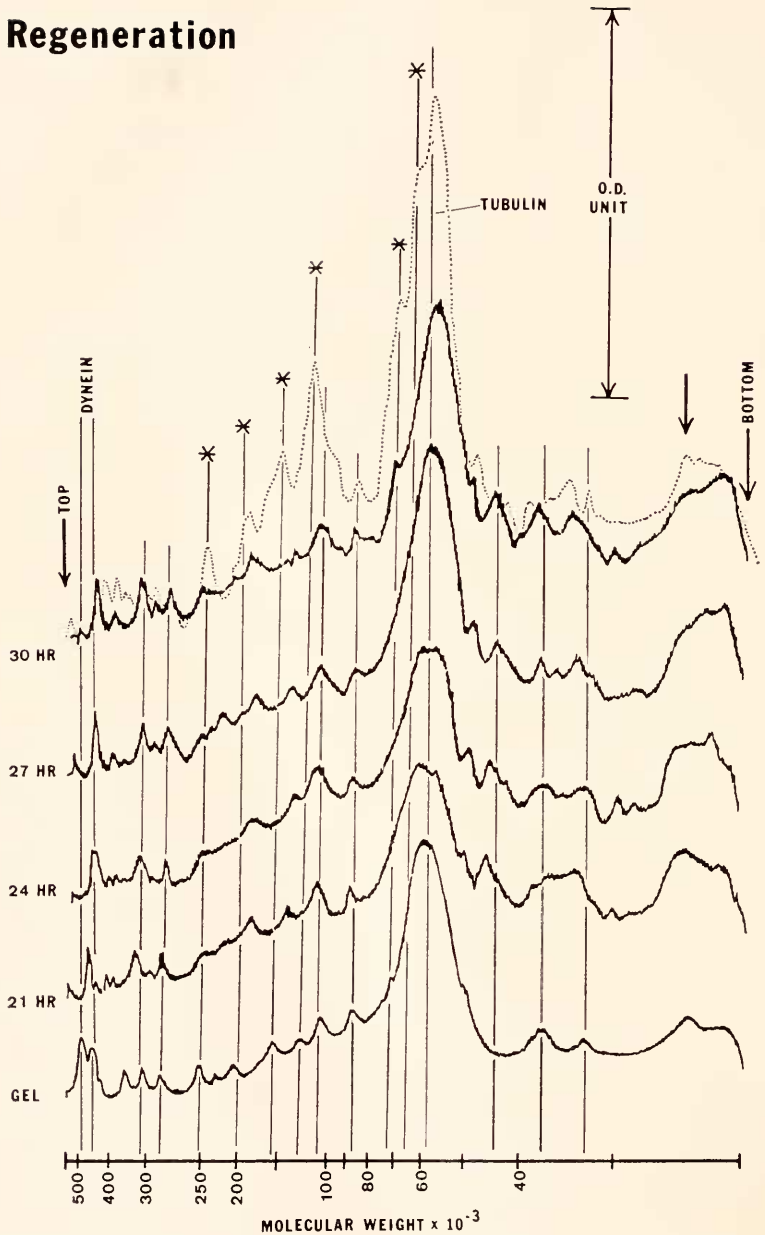

FIGURE 5. Incorporation of labeled proteins into ciliary axonemes upon regeneration from previously labeled and deciliated embryos. Microdensitometer tracings of gel-autoradiograms from regenerated ciliary axonemes. The bottom trace is from a stained gel from a 15–18 hour pulse period. The autoradiogram traces are nearly indistinguishable from one another, except for the higher amount of tubulin label in the 27–30 hour and 30–33 hour regenerates. The dotted trace is the upper trace from Figure 4, permitting direct comparison

of the markedly higher tubulin specific activity during the period of active ciliogenesis.

Discussion

The embryonic construction of a cilium begins well before fertilization with the synthesis of the high molecular weight enzymatic component of dynein. After fertilization the lower molecular weight component is synthesized at a constant rate throughout development. Various "background" proteins are also synthesized in a like manner. All of these components are apparently made well in excess over that needed for one generation of cilia since they have essentially the same specific activity in second generation cilia. Tubulin also shows a constant rate of synthesis up to early ciliogenesis and then increases by about 50%. One protein of molecular weight 300,000 appears to decrease in synthetic rate just prior to ciliogenesis.

Given this large pool of precursor material, some event or events must trigger ciliary morphogenesis at the prescribed moment. Perhaps most important in the scheme of assembly is the apparent *de novo* synthesis of "minor" structural components during active ciliogenesis. These presumably constitute the linkage-spoke complex that gives three-dimensional structure to the axoneme. Nearly all of these *de novo* components are utilized to form the virgin cilia since little label is found in these proteins upon regeneration. The *de novo* synthesis of the linkage complex as an initiating morphogenetic event is not inconsistent with the inhibition studies of Auclair and Siegel (1966); if synthesis of these minor constituents continued at the same rate after the initial ciliogenesis, there would be many "rounds" of these proteins available at the gastrula stage where continued multiple reciliation was demonstrated. Also, "90% inhibition" may still allow such components to be made, especially since ciliary proteins are apparently synthesized preferentially. In contrast to the studies of Auclair and Siegel (1966) and Auclair and Meismer (1965), Child and Apter (1969) demonstrated that growth or regeneration of cilia could be prevented by application of *Pactamycin* just before the scheduled onset of cilia formation. Here 94% inhibition of protein synthesis could be shown. Since protein synthesis was turned off only briefly prior to ciliogenesis, a time when the bulk structural components have already been synthesized, these authors propose that synthesis of a minor protein, critical for ciliary assembly, is inhibited, a conclusion not unlike that drawn from this present study.

The simultaneous operation of post-synthetic events to activate certain structural proteins cannot be ruled out. Mitotic tubulin, for example, can be differentially mobilized from an inactive form during prophase (Stephens, 1972b). It is not inconceivable that phosphorylation of bound GDP, intramolecular disulfide bond formation, or a specific cation addition might serve as "activation" steps, much as these three factors play an important role in the *in vitro* assembly of

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of virgin *versus* regenerated specific activities. The 21–24 hour and 24–27 hour regenerate tubulin is substantially lower than that from comparable time points in Figure 4, indicating roughly a 25% depletion of the labeled tubulin pool upon regeneration. Notation is the same as Figure 4.

flagellar B-tubulin (Stephens, 1971; in preparation). It might be pointed out here that the molecular weights of tubulin obtained from fertilized eggs, isolated mitotic apparatuses, and ciliary axonemes exhibit no obvious numerical differences, indicating that proteolysis is probably not an activation step. Still, specific proteolysis of components other than tubulin offers a reasonable method for mobilizing active structural building blocks from an inactive pool at a discrete time point in development.

One important basic biological question might be raised in light of the above data. If the tubulin and dynein components of the cilium are in abundance well before ciliogenesis and do not require "activation," what prevents them from forming doublet microtubules in conjunction with the mitotic centrioles? An essential morphogenetic event may be the *activation of the centriole*, enabling it to serve as a "crystallization center" for pre-existing and *de novo* components and thus form the basal body of the developing cilium. Perhaps Pickett-Heaps' (1969) argument that participation in mitosis simply ensures the centrioles of equal partitioning in the daughter cells is applicable here; they may be themselves nonfunctional until called upon in later development to serve as basal bodies.

The morphogenetic process in cilia is beginning to show some interesting parallels to other better-studied developmental processes. Sequential gene action, production of "minor" structural components, and subsequent modification of previously-synthesized proteins are well-established events in the morphogenesis of the phage T-4 (*cf.* Wood, Edgar, King, Lielausis and Henninger, 1968). The production of one "round" of *de novo* structural elements brings to mind a similar quantal production of enzymes in the developing cellular slime mold *Dictyostelium* (Newell, Longlands, and Sussman, 1971). Yet many critical questions remain to be answered. Paramount is the issue of the tubulin pool: how large is the pool prior to fertilization, what relative amount of total tubulin is synthesized post-fertilization, how many tubulins are involved at each stage, and into what structures are they incorporated? In addition, how (if at all) is ciliary tubulin "activated" *in vivo?* What is the role of the great excess of high molecular weight dynein component—a pool for ciliary regeneration or a mitotic ATPase? Is the centriole (basal body) unmasked as a "crystallizing center"? What is the nature of the unidentified *de novo* structural proteins and how are they involved in ciliogenesis? How is ciliary length determined? Is there some limiting component mobilized anew at each regeneration? Do micromeres, which never make cilia, synthesize the lower molecular weight dynein band or the *de novo* structural components characteristic of cilia? What is the pattern of ciliary protein synthesis *after* cilia formation and how is it affected by deciliation? Answers to these questions should provide a better understanding of the processes by which a developing embryo gives rise to its first self-generated organelle.

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Summary

1. Cilia were isolated from sea urchin blastula pulse-labeled with ^{14}C-leucine at various time points prior to and during ciliogenesis, the structural components fractionated by SDS-acrylamide gel electrophoresis, and the relative amount of labeled protein determined by autoradiography of gel slices.

2. The two components of the ciliary ATPase dynein are synthesized differentially. The higher molecular weight enzymatic component pre-exists before fertilization; only the lower molecular weight component is synthesized after fertilization and it is made at a constant rate.

3. A number of components with medium and low molecular weights are also synthesized uniformly throughout development. Of these, tubulin alone shows a marked increase in synthesis during late ciliogenesis. This protein is synthesized in three to four-fold excess over that needed for one generation of cilia. All of the others, including dynein, show no significant decrease in specific activity upon regeneration from previously labeled and deciliated embryos.

4. At the initiation of ciliogenesis, at least six minor components appear to arise *de novo* and only in sufficient amount for one generation of cilia.

5. These data support previous findings that the bulk of ciliary protein is made prior to ciliogenesis and in considerable excess, but suggest further that the morphogenetic process is marked by a "round" of *de novo* synthesis of minor but critical structural components.

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